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13. ABSTRACT (Maximum 200 Words)

The aims of this study are to identify the types and properties of telomerase producing cells within breast tumors, and further, to isolate these cells from breast tumors so that their biochemical and functional properties may be characterized. Through examining the role of telomerase in cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, epidemiology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work, are on schedule. The research is in progress.

14. SUBJECT TERMS
Real-time RT-PCR, telomerase, hTERT, in-situ hybridization (ISH), immunohistochemistry (IHC), fluorescence activated cell sorting (FACS)

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I. INTRODUCTION

The aims of this study are to identify the types and properties of telomerase producing cells within breast tumors, and further, to isolate these cells from breast tumors so that their biochemical and functional properties may be characterized. Through examining the role of telomerase in cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, epidemiology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work, have been initiated. The research is in progress and will extend beyond the timeline set forth in the Statement of Work.

Hypothesis and Rationale

To understand the effects of hTERT on breast cancer cell immortalization and tumorigenesis, it is necessary to study telomerase expression at the level of the individual cell. The preliminary experiments and the evidence in the current literature suggest that: 1) the levels of hTERT expression vary within the cellular subpopulations of a tumor and/or 2) the fraction of cells expressing telomerase varies between tumors. Based on these considerations, ***I hypothesize that breast cancer cells with the highest expression of hTERT will have the most aggressive phenotype.*** I will evaluate this hypothesis by testing the predictions in two specific aims.

- **Specific Aim #1**
  To identify the types, numbers, and properties of breast tumor cells expressing hTERT, so as to define the variability in cellular expression of hTERT in human breast tumors.

- **Specific Aim #2**
  To characterize the biochemical and functional properties of breast tumor cells expressing elevated hTERT, so as to determine if these cells have the more aggressive phenotype that is correlated with a poor clinical outcome.

II. Key Research Accomplishments

IIa. RESEARCH ACCOMPLISHMENTS

- The principal findings of ISH and Quantitiative RT-PCR are: 1) TERT expression is restricted to epithelial cells; 2) TERT is expressed in both normal and malignant tissues of the breast; 3) The pattern and level of TERT expression are heterogeneous in both tumor and normal tissues; and 4) Tumors with above-normal TERT mRNA levels are most likely to have an aggressive phenotype.

- An analysis of telomere content and allelic instability in tumor and normal breast tissue indicate that genomic instability occurs in histologically normal breast tissues adjacent to the corresponding tumors, and that the extent of this genomic instability is a function of distance from the visible tumor margins.

- Evaluation a single nucleotide polymorphisms with the angiotensin converting enzyme demonstrates that the "DD" genotype is associated with QTc prolongation, which suggests genetic factors affect QTc interval within End-Stage Renal Disease.

- Analysis within breast tumors of the telomere content, and the corresponding mRNA levels of telomere-associated proteins: Telomere Repeat binding Factor 1 and 2 (TRF1 & TRF2), TRF1 Interacting Nuclear protein 2 (TIN2), Protection of Telomeres 1 (POT1), and TERT demonstrate that the telomere content can be predicted within tumors by a mathematical model of these mRNAs encoding the telomere-associated proteins.

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the activation of this award the PhD candidate has had the opportunity to work and interact with oncologists, pathologists and other Ph.D. scientists who specialize in breast cancer. This has principally through tumor board meetings, journal clubs, special seminars and direct interaction within the laboratory. He has been trained by experts that oversee the Microscopy and Flow Cytometry core facilities at the UNM Health Sciences Center. The Ph.D. candidate's research is overseen by his research committee, a body comprised of three Ph.D. scientists with interests in breast cancer, and one M.D. that specializes in breast cancer pathology.

This predoctoral grant has allowed the candidate to gain technical expertise and develop a broad range of skills that will allow him to function independently. He has shown the ability to create, modify, and adapt scientific protocols according to his specific goals. He has acquired the analytical skills that will be required for him to further his career in science, and has demonstrated effective oral, written, and verbal communication skills as evidenced by his seminar and journal club presentations, poster presentations, lectures, research articles, and authored chapters. He plans to defend his dissertational work in the Summer of 2005.

IIc. <u>DETAILED SUMMARY OF PERFORMANCE ACCOMPLISHMENTS</u> (listed per FY2002-03 reviewer's comment: "List performance accomplishments in an outline based upon the statement of work.")

Specific Aim 1: (13 tasks)

Task 1 Month 1 **Complete**

- Identification of a sample breast tumor population consisting of low, intermediate, and high hTERT mRNA expressing tumors.

- The acquisition of human breast tissue is in progress as approved by our institution's Human Research and Review Committee (HRRC)

Task 2 Month 1-6 **Complete**

- DNA/RNA has been extracted from newly selected breast tumors.

Task 3 Month 1-6 **Complete**

- Quantitative RT-PCR analysis of hTERT mRNA on newly selected breast tumors

Task 4 Month 12-36 **In Progress**

- Cryosectioning of breast tumors

Task 5 Month 6-12 **Complete**

- Optimize hTERT FISH assay

Task 6 Months 12-18 **Complete**

- perform hTERT FISH assay

Task 7 Months 12-18 **Not Successful**

- Optimize hTERT IHC assay:
- We have determined that the current commercial antibodies to telomerase did not produce satisfactory results for immunohistochemical detection within frozen breast sections. Other researchers in the telomerase field have confirmed negative results using these same antibodies.

Task 8 Months 12-18 **Complete**

- Optimize cytokeratin-7 IHC assay

Task 9 Months 12-18 **Complete**

- Optimize vimentin IHC assay
- photo available in Appendix C.

Task 10 Months 12-18 **Complete**

- Optimize α-actin IHC assay

Task 11 Months 12-18 **Complete**

- Optimize common acute lymphocytic leukemia antigen IHC assays

Task 12 Months 18-20 **Initiated, not complete**
- Optimize IHC triple-labeling experiments

Task 13 Months 21-36 **Incomplete, not initiated**
- Perform IHC triple-labeling assay
- Analysis of the serial sections of tumors and tumor-associated normal breast histological sections were sufficient to provide required data.

Specific Aim 2: (5 tasks)

Task 1 Month 12-36 **In Progress**
- Creation of primary cell cultures from fresh breast tumors.
- Acquisition of samples from the University of New Mexico Pathology and Cooperative Human Tissue Network (CHTN) is in progress.

Task 2 Month 22-36 **In Progress**
- Growth analyses of primary cell cultures
- A photo of these cells is shown in appendix A

Task 3 Month 12-15 **Complete**
- Creation of GFP reporter plasmid (months 12-15)
- An Additional control adenovirus, Ad-CMV-DSRED has been created as a positive control (Not Shown).

Task 4 Month 15-36 **In Progress**
- Optimization and flow separation of cells based on GFP levels
- A figure of Flow separation is shown in Appendix B

Task 5 Month 15-36 **In Progress**
- Characterization of hTERT-rich cells by growth rate, anchorage independence, DNA flow cytometry, telomere content.

III. REPORTABLE OUTCOMES

Publications:

Peer Reviewed Publications:

Raizada V, Skipper B, Luo B,Garza I, Hines WC, Harford A, Zager P, Griffith, Raj D, Spalding CT. ACE DD Genotype and QTc Prolongation in End -Stage Renal Disease. Kidney International, In Press

Articles Submitted for Publication

Hines W.C., Fajardo AM, Joste NE, Bisoffi M, Griffith JK. Quantitative and Spatial measurements of TERT expression within normal and malignant human breast tissue. Submitted to Molecular Cancer Research

Christopher M. Heaphy, Marco Bisoffi, Colleen A. Fordyce, Christina M. Haaland-Pullus, William C. Hines, Nancy E. Joste, and Jeffrey K. Griffith. Telomere DNA Content and Allelic Imbalance in Histologically Normal Tissue Adjacent to Breast Tumors: Implications for Prognosis – Submitted to Cancer Research

Butler KS, Hines WC, Roberts D, Fordyce CA, Griffith JK. Levels of telomere protein mRNAs predict telomere content in human breast tumors (2005) Submitted to Cancer Research

Chapters in Books

William C. Hines, Robert H. Glew. Gaucher Disease; *Clinical Studies in Medical Biochemistry*, Third Edition. Oxford University Press.

Patent (Provisional):

Inventor(s): M. Bisoffi; J. Griffith; C. Heaphy; W. Hines. Tumor Assessment: A Simple, High-Throughput Method for Measuring the Extent of Genomic Instability in Tissue Samples. Reference number: MC-278.

Presentations: (abstracts in Appendix)

Hines W.C., Griffith JK Identification of the Types, Properties, and Functional Characteristics of Telomerase Expressing Cells in Breast Cancer. DOD Era of Hope Meeting, Philadelphia, PA, June 8-11 2005.

Hines W.C., Fajardo AM, Joste NE, Bisoffi M, Griffith JK. TERT expression within normal and malignant human breast tissue. University of New Mexico Biochemistry Research Day, April 23, 2005

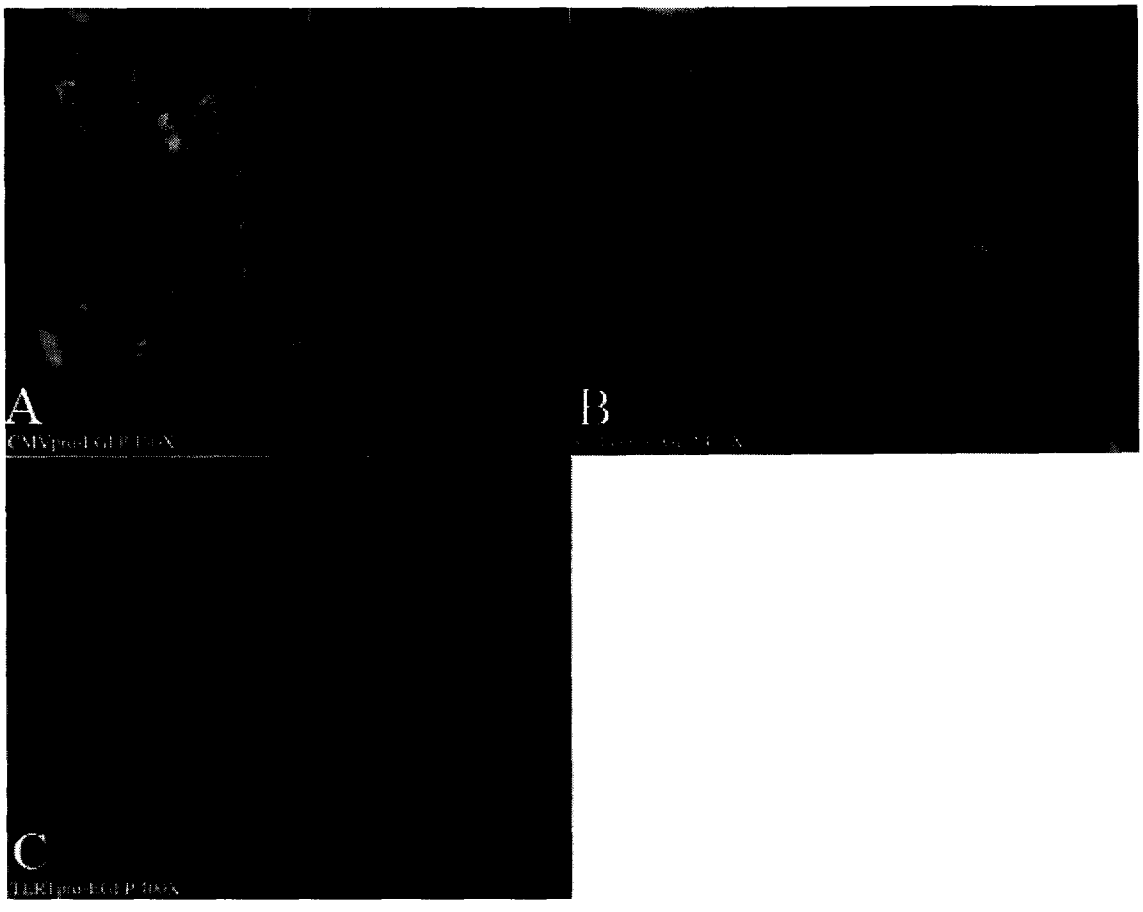
Christopher M. Heaphy, Colleen A. Fordyce, William C. Hines and Jeffrey K. Griffith. Telomere DNA Content in Cancerous and Proximal Histologically Normal Tissues Predicts Disease-free Survival in Breast Cancer Patients. 27th Annual San Antonio Breast Cancer Symposium. Dec 8th-11th, 2004

Christopher M. Heaphy, William C. Hines, Marco Bisoffi, and Jeffrey K. Griffith. Diagnosis and Prognosis of Cancer: A Simple High-Throughput Method for Measuring the Extent of Genomic Instability in Tissue Samples. Annual Iberoamerican Research and Development Summit (AIRDS), Nov 9-11 2004

IV. Conclusions

To date, all tasks; as outlined in the Statement of Work, have been completed or are in progress. A manuscript based on the results from this body of work has been submitted to Molecular Cancer Research, and the PI is a secondary author on three other manuscripts. The PI has demonstrated the ability to grow primary cells derived from breast tumors, and demonstrated their ability to 1) be infected with his adenoviral constructs and 2) be analyzed and sorted by Flow Cytometry.

APPENDIX A



Breast Tumor Organoids and Cellular Outgrowths infected with Adenoviral Promoter Constructs. Tumors were minced and placed within short-term cell culture. At Day 1 after collection, the cultures were infected with either an adenovirus encoding Green-Fluorescent Protein (GFP) under the control of the CMV promoter, Ad.CMV-EGFP (A and B); or a virus containing GFP under transcriptional control of the telomerase (TERT) promoter, Ad.TERTpro-EGFP (C).

APPENDIX B

ERA OF HOPE ABSTRACT:

IDENTIFICATION OF THE TYPES, PROPERTIES, AND FUNCTIONAL CHARACTERISTICS OF TELOMERASE EXPRESSING CELLS IN BREAST CANCER

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Genomic instability is a widespread and common feature of most, if not all, neoplasms, and the cell's loss of telomere function is a notable mechanism generating instability. Telomeres are protein-DNA complexes that protect chromosome ends from degradation and recombination. Due to a DNA replication machinery problem, the cell loses up to 50 to 200 bases of distal telomeric DNA during each cell division; a complication known as the "End-Replication-Problem." Telomere shortening may also result from double-strand DNA breaks, or changes in either the expression or function of any of several proteins required for telomere maintenance. Within dividing cells, the loss of telomere DNA ultimately results in cellular senescence and eventually, cell death. This loss; however, may be balanced through the activity of telomerase, the specialized reverse-transcriptase that synthesizes telomere repeats.

It is widely believed that nearly all tumors express telomerase, suggesting potential roles for telomerase in cancer diagnosis and treatment. However, the effect of cellular heterogeneity of telomerase expression is poorly understood.

The purpose of this study was to compare the expression of hTERT, the catalytic subunit of telomerase, in human breast tissues by RT-PCR and in situ hybridization. The levels of hTERT mRNA were measured by real time RT-PCR in 36 breast carcinomas and 5 samples of normal breast tissue. The levels of hTERT mRNA varied 750-fold in tumors, although 75% of tumors had hTERT levels that were statistically equivalent to the normal tissues. Tumors that had hTERT mRNA levels greater than those of normal tissues were more likely to be histological grade III tumors (0.002), have a high S-phase fraction (0.004), and contain an increased level of cmyc mRNA (p=0.034).

The sites of hTERT expression were determined by fluorescent in situ hybridization. We find that: 1) The expression of hTERT mRNA was specific to the epithelial cells in both normal and malignant tissue, 2) The cellular expression of hTERT is higher within the normal epithelial cells than within cells from the majority of tumors, and 3) The sites and levels of hTERT expression were heterogeneous in both tumor and normal tissues. For example, there was strong expression of hTERT within the cells forming the terminal lobular duct of normal tissue, which was absent in adjacent cells within the same lobular unit. Likewise, in tumor tissue, there were fields of cells strongly expressing hTERT, whereas neighboring cells had distinguishingly different staining intensities.

These findings indicate that telomerase expression is regulated within the normal breast, breast tumors develop in a cellular environment characterized by expression of telomerase, and telomerase expression appears to be down regulated, on a per cell basis, in a majority of tumors. However, the subset of tumors that express the highest levels of hTERT have more aggressive characteristics.

APPENDIX C

TERT expression within normal and malignant human breast tissue.

University of New Mexico Biochemistry Research Day, April 23, 2005
Hines W.C., Fajardo AM, Joste NE, Bisoffi M, Griffith JK.

Abstract

The enzyme telomerase catalyzes the de novo synthesis of telomere repeats, thereby maintaining telomere length, which is necessary for unlimited cellular proliferation. Telomerase Reverse Transcriptase (TERT), the catalytic domain of telomerase, is the rate-limiting factor for telomerase activity and expressed in virtually all tumors. Thus, TERT has been proposed as a marker with diagnostic and prognostic potential in breast cancer, as well as a basis for breast cancer therapeutics. In these contexts, it is important to define the sites and extent of TERT expression with the normal and cancerous human breast tissues. In this study, levels of TERT mRNA were measured within a set of 36 breast carcinomas and 5 normal breast samples by quantitative real-time RT-PCR (QRT-PCR), and subsequently identified and characterized the cells expressing TERT mRNA within these tissues using *in situ* hybridization. The results demonstrate that (i) TERT mRNA expression is specific to the epithelial cells, (ii) TERT is expressed in both normal and malignant breast tissues (iii) the pattern and level TERT expression are heterogeneous, with nearly 75% of tumors expressing bulk TERT mRNA levels equal to, or less than those, within normal breast tissue; and (iv) tumors expressing above-normal levels of TERT mRNA were more likely to be histopathological grade III ($p=0.002$), contain high fraction of cells in S-phase ($p=0.004$), and have increased levels of MYC mRNA ($p=0.034$).

APPENDIX D

27th Annual San Antonio Breast Cancer Symposium

Telomere DNA Content in Cancerous and Proximal Histologically Normal Tissues Predicts Disease-free Survival in Breast Cancer Patients.

Christopher M. Heaphy, Colleen A. Fordyce, William C. Hines and Jeffrey K. Griffith.

ABSTRACT

Background: Telomeres are specialized nucleoprotein complexes that protect and stabilize the ends of linear chromosomes. Telomere attrition, induced for example by incomplete DNA replication during mitosis, is a prime source of genomic instability and a hallmark of cancers, including breast cancer. Because genomic instability leads to phenotypic variability, which in turn drives the development of aggressive cell clones, we hypothesized that telomeric DNA content (TC) predicts clinical outcome in breast cancer patients.

Material and Methods: The present study included two independent cohorts of breast cancer specimens. The first cohort (n=29) was a case-control group consisting of large, node-positive tumors; for this group, proximal histologically normal (PHN) tissue was also available. The second cohort (n=54) consisted of randomly selected invasive ductal carcinomas. TC was measured using a chemiluminescence hybridization assay, and allelic imbalance (AI) was determined by multiplex PCR analysis of 16 genome-wide unlinked microsatellites. Associations between TC and either AI or disease recurrence were analyzed by Wilcoxon/Kruskal Wallis Rank Sums test; associations between TC and time of disease-free survival were determined by Kaplan/Meier Log Rank analysis.

Results: The extent of AI was associated with TC in both breast cancer study groups ($p=0.003$ and $p=0.015$). In the first group, TC was associated with disease recurrence within 84 months of surgery ($p=0.012$) and with time of disease-free survival ($p=0.017$). TC was also associated with recurrence status and time of disease-free survival in PHN tissue ($p=0.025$ and $p=0.006$).

Conclusions: Our data indicate that (i) telomere attrition occurs early in fields of breast tissues, including histologically normal areas; (ii) leads to genomic instability, which represents a fertile ground for the malignant transformation of epithelial cells. More importantly, our study shows that TC is a prognostic marker of disease recurrence in breast cancer. Thus, TC has the potential to identify breast cancer patients at high risk for disease progression and could be used in the clinical management of breast cancer.

APPENDIX E
Annual Iberoamerican Research and Development Summit (AIRDS), Nov 9-11 2004

Diagnosis and Prognosis of Cancer: A Simple High-Throughput Method for Measuring the Extent of Genomic Instability in Tissue Samples.

Christopher M. Heaphy, William C. Hines, Marco Bisoffi, and Jeffrey K. Griffith.

CONCEPT / INNOVATION DESCRIPTION

- There is a high demand for reliable molecular markers and assays able to detect cancer (DIAGNOSIS), as well as to discriminate between indolent and aggressive tumors (PROGNOSIS). Such assays will improve the clinical management and decision making for cancer patients.

- We have developed a simple, high-throughput molecular assay that quantifies the extent of allelic imbalance in human tissues. Allelic imbalance is an indicator of genomic instability, which affects whole chromosomal regions. It is a general characteristic of malignancies, as well as an indicator of disease recurrence (metastatic disease).

- The assay uses commercially available reagents, instrumentation, and analysis software. Our invention is based on a NOVEL INTERPRETATION method of the generated data, which can be used in the DIAGNOSIS and PROGNOSIS of ALL TUMOR types, regardless of their origin.

- Because of its simplicity and suitability for clinical laboratories, we expect the assay to have a high market impact.